

Supporting Information

Tian *et al.* 10.1073/pnas.0806700106

SI Methods

Channel Constructs. The generation of full-length murine ZERO and STREX channel epitope-tagged as well as TEA-pore mutant (Y334V) constructs have been described (14, 31). To generate the C-terminal –GFP fusion proteins a forward primer containing a KpnI restriction site, with a kozak consensus sequence immediately upstream of a new ATG start site was used to engineer an amplicon in which I323 was mutated to the new start methionine. The amplicon was digested with KpnI and PacI and ligated into the KpnI-PacI-digested full-length STREX-GFP or ZERO-GFP channel in pcDNA3.1. This generates a –GFP fusion protein of the entire STREX or ZERO channel C terminus with the start sequence now MIELIG.... To generate the CRD constructs forward primers with an XhoI site and a reverse primer with a BamHI site were used to amplify the STREX or ZERO CRD such that the start sequence in both constructs is CKACH. The PCR amplicon was digested with XhoI and BamHI and ligated into the XhoI and BamHI site of mCerulean (mCer) so that mCerulean is an N-terminal in-frame fusion with the respective CRD. An NheI-BamHI fragment of the mCer-CRD construct was then subcloned into pEYFP-N1 to generate the respective mCer-CRD-YFP construct with an in-frame N-terminal mCer and C-terminal YFP fusion. A single –YFP fusion of the STREX CRD was also generated using a forward primer to incorporate a kozak sequence and start methionine N-terminal to CKACH... The mCer-CRD, CRD-YFP and mCer-CRD-YFP constructs displayed identical behavior. All mutagenesis was performed using Quickchange mutagenesis (Stratagene) with constructs fully sequenced on both strands to verify sequence integrity.

Imaging. Briefly, cells were plated on glass coverslips, transfected as above and fixed 48 h after transfection. Cells were first washed

twice with PBS (Invitrogen) then fixed with ice-cold 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature. Cells were washed 3 times with ice-cold PBS and quenched with 50 mM NH₄Cl in PBS for 10 min. Cells were washed 3 times in ice-cold PBS before mounting on microscope slides using Mowiol. Cells were initially analyzed under epifluorescence using an inverted Nikon Eclipse 2000 microscope using a 100× oil objective lens. Confocal images were acquired on a Zeiss LSM510 laser scanning microscope, using a 63× oil Plan Apo-chromat (N.A. = 1.4) objective lens, in multitracking mode to minimize channel cross-talk. For each independent cell transfection 3–4 coverslips were analyzed for each construct. For each coverslip 3–5 random fields of view were analyzed to determine the number of transfected cells with plasma membrane localization of the respective fusion protein. The average percentage of transfected cells from each well was then determined for each independent transfection (experiment) and normalized to the corresponding wild-type STREX control (membrane expression was typically observed in >95% of transfected wild-type STREX fusion proteins). The majority of experiments were performed blind by an observer independent from the experimentalist undertaking the cell transfections. In addition, line scans of fluorescent intensity through 4 independent areas of the plasma membrane, cytosol and nucleus were analyzed in the LSM browser (Zeiss) or Volocity (Improvision) software to determine plasma membrane expression of fusion proteins. A signal intensity at the plasma membrane 2 SD greater than that in the cytosol in any of the 4 planes was defined as a cell with membrane expression. Data are shown as mean ± SEM for *N* independent experiments where *n* = minimum total number of cells analyzed across experiments for each construct.

14. Tian L, *et al.* (2004) Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 101:11897–11902.

31. Chen L, *et al.* (2005) Functionally diverse complement of large conductance calcium- and voltage-activated potassium channel (BK) alpha-subunits generated from a single site of splicing. *J Biol Chem* 280:33599–33609.

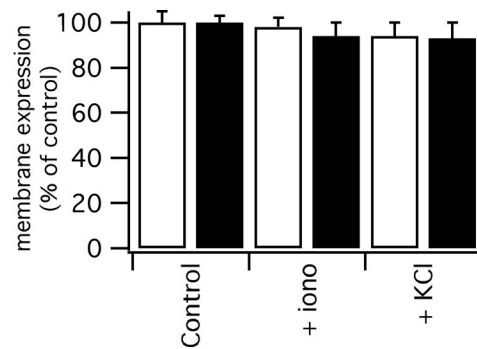
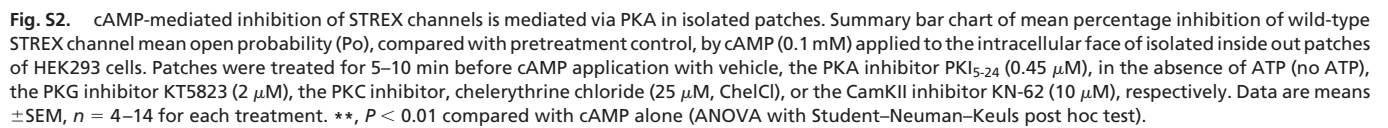


Fig. S1. Ionomycin induced calcium elevation and KCl-depolarization do not affect STREX membrane localization. Effect of ionomycin- ($1\ \mu\text{M}$, + iono) induced calcium elevation or 25 mM KCl depolarization (10–30 min) on STREX C terminus (■) or CRD (□) membrane localization in HEK293 cells. Data are expressed as the percentage relative to the respective vehicle-treated STREX control as means \pm SEM, $n > 4$, $n > 350$.



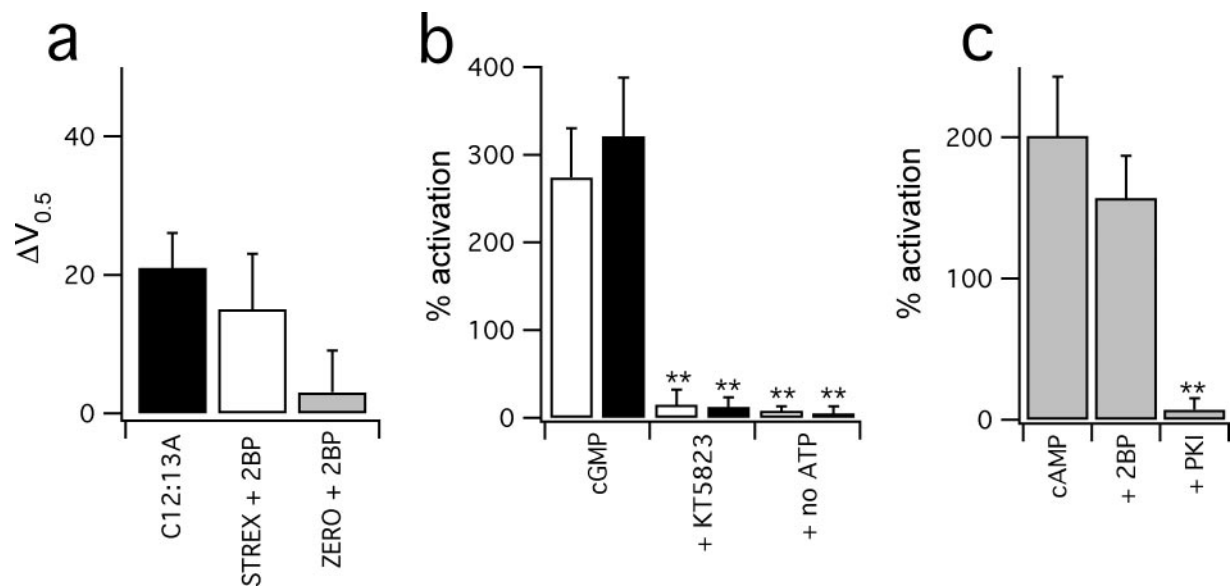


Fig. S3. Effect of palmitoylation status on $V_{0.5}$ and regulation of BK channel activation by PKG or PKA. (a) Summary bar chart of the change in voltage for half maximal activation ($\Delta V_{0.5}$) of the C12:13A mutant (■), and STREX (□) or ZERO (▒) channels treated overnight with the palmitoyl transferase inhibitor, 2-BP (100 μ M). Data are means \pm SEM, $n = 7-8$ per group. (b) Summary bar chart of the effect of PKG activation (0.1 mM cGMP) in isolated inside-out patches from HEK293 cells expressing the STREX (□) or C12:13A mutant (■). Data are the mean activation in 12 of 15 patches for STREX and 6 of 7 patches for C12:13A. The effect of cGMP was abolished in the presence of the PKG inhibitor KT5823 (2 μ M, $n = 3-4$ per group) or in the absence of ATP ($n = 3$ per group). (c) Summary bar chart of the stimulatory effect of cAMP (0.1 mM) in isolated inside-out patches from HEK293 cells expressing the ZERO variant (▒) from vehicle- ($n = 6$) and 2-BP-treated ($n = 5$) cells or in patches exposed to the PKA inhibitor PKI₅₋₂₄ (0.45 μ M). Data are means \pm SEM. **, $P < 0.01$ compared with respective stimulatory effect of cGMP (b) or AMP (c) alone (ANOVA with Student–Neuman–Keuls post hoc test).

Table S1. Predicted palmitoylated cysteine residues in STREX BK channel

Position	Peptide	CSS-palm v2.0		CSS-palm v1.0		Location
		Entire CDS	STREX insert	Entire CDS	STREX insert	
14	EVPCDSR	1.34	na	<2.6	na	Extracellular
53	WTVCCHC	1.55	na	2.80	na	Intracellular (S0-S1linker)
54	TVCCHCG	1.48	na	2.90	na	Intracellular (S0-S1linker)
56	CCHCGGK	0.92	na	2.78	na	Intracellular (S0-S1linker)
141	IESCQNF	1.10	na	<2.6	na	Extracellular
557	CELCFVK	0.87	na	<2.6	na	Intracellular (C-terminus)
STREX C12	RRACCFD	2.48	5.38	<2.6	<2.6	Intracellular (C-terminus)
STREX C13	RACCFDC	0.57	2.87	4.77	4.77	Intracellular (C-terminus)
STREX C16	CFDCGRS	1.08	2.87	3.69	3.69	Intracellular (C-terminus)
STREX C51	VNDCTSF	<0.5	0.65	3.00	3.00	Intracellular (C-terminus)
855	INLCDMC	0.80	na	2.69	na	Intracellular (C-terminus)
858	CDMCVIL	0.82	na	<2.6	na	Intracellular (C-terminus)
1059	DLFCKAL	<0.5	na	2.69	na	Intracellular (C-terminus)

Scores indicate CSS-palm prediction; higher number represents higher probability. CSS-palm scores were determined with the published CSS-palm v1.0 algorithm and the recently revised CSS-palm v2.0 algorithm at http://bioinformatics.lcd-ustc.org/css_palm/prediction.php by inputting either the full-length coding sequence (CDS, start methionine MDALLI set to residue number 1) for the murine STREX BK channel or for the STREX insert sequence alone. Predicted palmitoylated cysteine residues are indicated in bold with the 3 immediately flanking N- and C-terminal residues. Only residues with high probability scores (highest cutoff) in either v1.0 or v2.0 of CSS-palm prediction are included, and cysteines predicted to be at the intracellular aspect of the channel are indicated here. na, not applicable because not in input sequence.